Inhibition of leucocyte elastase by heparin and its derivatives

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Leucocyte proteinases, e.g. leucocyte elastase and cathepsin G, are inhibited by heparin. The activities of pig pancreatic and *Pseudomonas aeruginosa* elastases are unaffected by this polysaccharide. Heparin derivatives of known M_r and degree of sulphation were isolated. The inhibition of leucocyte elastase by these oligosaccharides can be classified as tight-binding hyperbolic non-competitive. K_i values ranged from 40 nM to 100 μ M and were found to be inversely correlated with the chain length of the oligosaccharides. Desulphated compounds lacked inhibitory potential towards leucocyte elastase. Over-*O*-sulphated di- and tetra-saccharides are more potent inhibitors than their over-*N*-sulphated counterparts. It is proposed that the therapeutic use of heparin and its derivatives could be extended to disease states such as emphysema and rheumatoid arthritis, where the role of leucocyte elastase has been clearly established.

INTRODUCTION

Leucocyte elastase (EC 3.4.21.37) is a potent mediator of connective-tissue breakdown, since it is able to degrade several of its macromolecular constituents under physiological and experimental conditions (Starkey & Barrett, 1976; Starkey, 1977; Bieth, 1978); the inefficient control of this proteinase by naturally occurring inhibitors may lead to disease states such as emphysema and rheumatoid arthritis (Janoff, 1978). In order to overcome the elastase/elastase inhibitor imbalance, the use of specific exogenous inhibitors has been proposed (Powers, 1983; Snider *et al.*, 1985). The major drawbacks of this approach may reside in the possible undesirable side effects of such inhibitors (Lucey & Stone, 1982).

Leucocyte elastase is a highly basic protein, with pI near 10 (Starkey, 1977), and it has been shown that several glycosaminoglycans could inhibit this proteinase via electrostatic interactions (Baici *et al.*, 1980; Baici & Bradamante, 1984; Laghi Pasini *et al.*, 1984; Lentini *et al.*, 1985). Heparin, which belongs to this class of compounds, is a complex and heterogeneous mixture of polysaccharide chains that vary in M_r and degree of sulphation (Jaques, 1980). These heparin fractions differ in their interaction with physiologically important proteins such as antithrombin III (Rosenberg, 1977) and Factor Xa (Engelberg, 1984). Heparin also exhibits several other biological functions, and its beneficial use in the prophylaxis and therapy of atherosclerotic disease is well documented (Engelberg *et al.*, 1965; Telford & Wilson, 1981).

In the present paper we provide evidence that heparin is a potent inhibitor of leucocyte elastases from rat and human origins; it can also inhibit human cathepsin G, but to a lesser extent. The mechanism of the inhibition of leucocyte elastase by heparin was studied with heparin fractions and fragments differing in their M_r and degree of sulphation.

MATERIALS AND METHODS

Heparin and its derivatives

Pig mucosal standard heparin [160 U.S.P. units/mg; 155 anti-(Factor XA) units/mg] came from Institut Choay (Paris, France). CY 222 polysaccharides (M_r 2500–8000) were obtained by chemical depolymerization of heparin with HNO_2 and gel filtration (Bâzu *et al.*, 1986). Homogeneous oligosaccharides were obtained from CY 222 polysaccharides by gel filtration on a Sephadex G-50 (superfine grade) column (300 cm $\times$ 2.5 cm) eluted with 0.2 M-NaCl. The degree of polymerization of the compounds was deduced from analysis of the chromatograms and confirmed by ^{13}C -n.m.r. analysis. They were desalted by gel filtration on Sephadex G-25. The M_r values of heparin fragments were determined by h.p.l.c. on either TSK SW 2000 (Toyo, Soda, Japan) or Lichrospher Si 100 (Merck, Darmstadt, Germany) columns. The columns were calibrated with standards whose M_r values had been determined by ultracentrifugation (Barlow, 1983). Their degree of sulphation ($\text{SO}_3^-/\text{CO}_2^-$) was determined by conductimetry by the procedure of Casu & Gennaro (1975). These oligosaccharides are referred to as the first series (series I) in our experiments.

Their degree of sulphation was increased by *O*-sulphation of their corresponding quaternary ammonium salts in organic solvents (series II); the oligosaccharides from the third series (series III) were obtained by *N*-desulphation and *N*-acetylation of the oligosaccharides of series I; finally, the oligosaccharides of the fourth series (series IV) were obtained by *O*-sulphation of the oligosaccharides of series III.

Enzymes and assays

Rat leucocyte elastase was purified from rat pleural exudates by κ -elastin-agarose affinity chromatography and CM-cellulose ion-exchange chromatography as

Abbreviations used: Suc-, 3-carboxypropionyl-; -NH-Np, *p*-nitroanilide.

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Fig. 2. Inhibition of rat leucocyte elastase by oversulphated tetra- (●), octa- (○) and hexadeca- (▲) saccharides derived from heparin

For experimental details see the text. The substrate used was Suc-Ala-Ala-Ala-NH-Np (1.25 mM). The dotted line corresponds to the theoretical curves drawn by applying the equation:

$$\frac{v_0}{v_i} = \frac{1 + [I]/K_i}{1 + \beta[I]/K_i}$$

and the β and K_i values are determined by Fig. 4; see also Table 1.

elastases and human cathepsin G are inhibited by low concentrations of heparin. At inhibitor concentrations greater than 0.5 ng/ml the maximal inhibitions of rat and human leucocyte elastases are similar, e.g. 90%; in contrast, a maximal inhibition of only 45% was obtainable with human cathepsin G at similar heparin concentrations. For all these enzymes the shape of plots of reciprocal velocity versus heparin concentration were hyperbolic.

As has also been hypothesized for Arteparon and other glycosaminoglycans (Baici *et al.*, 1980; Lentini *et al.*, 1985), the interaction between leucocyte elastase and heparin probably involves the negatively charged sulphate groups of heparin and the positively charged guanidinium groups of the arginine residues of leucocyte elastase located at the surface of the molecule (Bode *et al.*, 1986; Baici, 1987). Leucocyte elastase and cathepsin G are very basic proteins, with pI 9–10 (Starkey, 1977; Starkey & Barrett, 1976). We found that ionic strengths higher than 0.5 completely abolished the inhibitory capacity of heparin towards leucocyte proteinases. In order to substantiate further the importance of sulphate groups in the inhibitory capacity of heparin derivatives towards leucocyte proteinases, several heparin derivatives of various M_r values were isolated and their degrees of sulphation were modified.

N-Desulphated *N*-acetylated octa- and hexadecasaccharides lack inhibitory potential towards rat leucocyte elastase. These compounds were also devoid of activity on human leucocyte elastase and cathepsin G. The corresponding oversulphated octa- and hexadecasaccharides are inhibitors of these enzymes. Fig. 2

Fig. 3. Influence of the Suc-Ala-Ala-Ala-NH-Np concentration ([S]) on the inhibition of rat leucocyte elastase by the oversulphated hexadecasaccharide (series II)

For experimental details see the text. Three substrate concentrations were used: 0.865 mM, 1.73 mM and 3.46 mM. K_m of rat leucocyte elastase for this substrate was equal to 2.5 mM.

illustrates that the extent of inhibition of rat leucocyte elastase depended upon the chain length of the heparin-derived oligosaccharides, similarly to observations with Arteparon fractions (Baici *et al.*, 1980).

The mechanism of inhibition of rat leucocyte elastase by heparin derivatives was studied with Suc-Ala-Ala-Ala-NH-Np as substrate; catalytic amounts of enzyme at several different substrate and inhibitor concentrations were used, and specific velocity (v_0/v_i) was plotted as a function of $([S]/K_m)/(1 + [S]/K_m)$.

[S] refers to the substrate concentration, and the K_m of rat leucocyte elastase for this synthetic substrate was 2.5 mM. Fig. 3 shows that the above representation resulted in a family of parallel lines corresponding to the different concentrations of the oversulphated hexadecasaccharide heparin derivative. Similar patterns were obtained with heparin and the oversulphated octasaccharide (series II), and are characteristic of tight-binding non-competitive hyperbolic inhibition, e.g. $\alpha = 1$ and $0 < \beta < 1$.

We therefore analysed our data according to the general equation proposed by Baici (1987) (Fig. 4). When an enzyme concentration equal to 80 nM was used for the calculation, a curve was obtained; $[E]_i$ was then adjusted to 100 nM by the trial-and-error procedure in order to obtain a linear representation.

This allowed us to determine the K_i and the β parameter for the oversulphated hexadecasaccharide heparin derivative. If the molar concentration of the compound is considered, K_i was equal to 73 nM and β was 0.11. K_i values were similarly evaluated for several heparin derivatives belonging to series I, II and IV (Table 1). The inhibition constants ranged from 100 μ M to 40 nM and were inversely correlated with the M_r of the heparin derivative in series I and II. With the exception of the hexadecasaccharide heparin derivative, oversulphation of the original polysaccharides led to compounds having greater inhibitory capacity (lower K_i) towards leucocyte elastase. It also appears from Table 1 that not all ester sulphates had the same importance; this became evident when comparing K_i values of the different tetrasaccharide derivatives respectively equal to 100 μ M (series I), 76 μ M (series II) and 16.7 μ M (series IV). *N*-Desulphated over-*O*-sulphated tetra-, hexa- and octa-saccharides also

Fig. 4. Inhibition of rat leucocyte elastase by the oversulphated hexadecasaccharide (series II)

For experimental details see the text. The M_r of the oligosaccharide was 4800 and the molar concentration of the compound was used for calculation; duplicate measurements were performed at three substrate concentrations (Suc-Ala-Ala-Ala-NH-Np: 0.865, 1.73 and 3.46 mM). $[I]_t$ represented the molar concentration of the oligosaccharide and $[E]_t$ the concentration of active-site-titrated leucocyte elastase. $[E]_t = 80$ nM: a curved plot was obtained. $[E]_t$ adjusted to 100 nM by trial-and-error procedure: $K_i = 73$ nM; $\beta = 0.11$.

Table 1. Inhibition of rat leucocyte elastase by heparin derivatives varying in chain length and degree of sulphation

For experimental details see the text.

	No. of disaccharide units	Degree of sulphation ($\text{SO}_3^-/\text{CO}_2^-$)	K_i (μM)
Series I	2	1.71	100
	5	1.94	23
	8	1.99	0.04
Series II	2	2.62	76
	3	2.69	16
	4	2.57	3.9
	5	2.57	5.83
	6	2.61	0.6
	8	2.62	0.073
Series IV	2	2.17	16.7

exhibited higher inhibitory potential as compared with their original counterparts with similar degree of sulphation but containing *N*-sulphate groups.

DISCUSSION

As with other glycosaminoglycans, e.g. Arteparon, chondroitin sulphates, dextran and chitosan sulphates,

heparin is also able to inhibit leucocyte elastase (Baici *et al.*, 1980; Baici & Bradamante, 1984; Lentini *et al.*, 1985). The activity of cathepsin G, another major proteinase of polymorphonuclear leucocytes (Starkey & Barrett, 1976), was also affected by heparin, although to a lesser extent. Oligosaccharides obtained by HNO_2 depolymerization of heparin were isolated and their sulphate contents were chemically modified in order to correlate the elastase-inhibitory capacity of these compounds to certain of their structural features such as their degree of sulphation and chain length. The mechanism of inhibition of leucocyte elastase by these heparin derivatives was first investigated by using the specific velocity plot method of Baici & Bradamante (1984), as well as the general equation more recently proposed by Baici (1987). Analysis of the data clearly established the tight-binding non-competitive hyperbolic nature of the inhibition. Arteparon fractions and heparin derivatives of similar average M_r have comparable K_i values; heparin derivatives, however, exhibited lower values of the β coefficients.

It was conclusively shown that the ability of heparin-derived oligosaccharides to inhibit leucocyte elastase was inversely correlated to the chain length of these substances. Although a minimal degree of sulphation was a prerequisite for these oligosaccharides to inhibit leucocyte elastase, it appeared that *O*-sulphated compounds exhibited lower K_i values as compared with their *N*-sulphated counterparts of similar M_r value. Of importance was the finding that the tetrasaccharide of series IV (*N*-desulphated over-*O*-sulphated oligosaccharides) possessed a K_i in the $10 \mu\text{M}$ range. The therapeutic activity of heparin is essentially due to its binding to antithrombin, thus accelerating the rate of neutralization of the proteolytic enzymes involved in the haemostatic mechanism (Lindahl *et al.*, 1984). Studies by Atha *et al.* (1985) demonstrated that the active site of heparin reacting with antithrombin is a pentasaccharide containing an *N*-sulphated and a 3-*O*-sulphated glucosamine residue. It is well established that both the degree of its sulphation and its M_r mainly influence the anticoagulant activity of heparin (Engelberg, 1984). Low- M_r heparin is more effective in inhibiting the activation of Factor Xa than in potentiating the action of antithrombin III towards thrombin.

Although inhibitory constants (K_i) are essential to evaluate the potency of an elastase inhibition, other parameters need to be taken into account, as for instance the ability of the compound to inhibit elastase pre-adsorbed on its substrate (Robert *et al.*, 1974; Hornebeck & Schnebli, 1982). We found that the oversulphated hexadecasaccharide (series II) inhibited leucocyte elastase equally well in its free state and adsorbed on elastin fibres (not shown).

The disruption of the integrity of the aortic endothelial layer is one of the main factors in atherogenesis (Telford & Wilson, 1981). Heparin binds and inactivates many of the substances that injure endothelium, including histamine, 5-hydroxytryptamine (serotonin), bradykinin, angiotensin and many toxins (Engelberg, 1984; Jaques, 1979). It was also demonstrated that aggregated leucocytes adhere to endothelial cells and can further damage the endothelial layer (Saba *et al.*, 1978). This damage could be partly a proteinase-mediated event, and in such a case the modulation of the activities of leucocyte elastase and cathepsin G by heparin and its derivatives could be determinant.

It appears therefore that the above-mentioned heparin derivatives, which have only low anticoagulant activity (Bâzu *et al.*, 1986), are of potential interest for the control of diseases where elastase/antielastase balance is upset in favour of leucocyte elastase.

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